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Tn-seq of *Caulobacter crescentus* under uranium stress reveals genes essential for detoxification and stress tolerance.

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ABSTRACT:

The ubiquitous aquatic bacterium *Caulobacter crescentus* is highly resistant to uranium (U) and facilitates U biomineralization, and thus holds promise as an agent of U bioremediation. To gain an understanding of how *C. crescentus* tolerates U, we employed transposon mutagenesis paired with deep sequencing (Tn-seq) in a global screen for genomic elements required for U resistance. Of the 3879 annotated genes in the *C. crescentus* genome, 37 were found to be specifically associated with fitness under U stress, 15 of which were subsequently tested through mutational analysis. Systematic deletion analysis revealed that mutants lacking outer membrane transporters (*rsaF$_a$* and *rsaF$_b$*), a stress-responsive transcription factor (*cztR*), or a ppGpp synthetase/hydrolase (*spoT*) exhibited a significantly lower survival rate under U stress. RsaF$_a$ and RsaF$_b$, which are homologues of TolC in *E. coli*, have previously been shown to mediate S-layer export. Transcriptional analysis revealed upregulation of *rsaF$_a$* and *rsaF$_b$* by 4- and 10-fold, respectively, in the presence of U. We additionally show that *rsaF$_a$* mutants accumulated higher levels of U compared to wild type, with no significant increase in oxidative stress levels. Our results suggest a function for RsaF$_a$ and RsaF$_b$ in U efflux and/or maintenance of membrane integrity during U stress. In addition, we present data implicating CztR and SpoT in resistance to U stress. Together, our findings reveal novel gene targets that are key to understanding the molecular mechanisms of U resistance in *C. crescentus.*

IMPORTANCE:

Caulobacter crescentus is an aerobic bacterium that is highly resistant to uranium (U) and has great potential to be used in U bioremediation, but its mechanisms of U resistance are poorly understood. We conducted a Tn-seq screen to identify genes specifically required for U resistance in *C. crescentus*. The genes that we identified have previously remained elusive using other omic approaches and thus provide significant insight into the mechanisms of U resistance by *C. crescentus*. In particular, we show that outer membrane transporters $RsaF_a$ and $RsaF_b$, previously known as part of the S-layer export machinery, may confer U resistance by U efflux and/or maintaining membrane integrity during U stress.

INTRODUCTION:

Uranium (U) contamination is widespread and imposes significant concerns to environmental ecology and human health (1). Chemical and physical techniques for waste treatment or removal of U are challenging and expensive. A promising, microbially-mediated method for U remediation, *in situ* U immobilization, is more cost effective and environmentally friendly than conventional approaches (2). If we seek to task microbes with the cleanup of contaminated sites, an understanding of how microbes defend against U toxicity is crucial. Understanding these mechanisms is necessary for the optimization of strains intended for use as U biosensors (3) or for the purpose of bioremediation (2, 4, 5).

A great deal is known about various strategies used by microbes to transform and defend against extracellular U. These include reductive precipitation by outer membrane cytochromes, conductive pili, or spores (6-10), surface adsorption by EPS or S-layers (9, 11), or precipitation with phosphate (4, 5, 12, 13). However, mechanisms used by cells for combating *internal* U toxicity are poorly understood (14). Previous studies revealed upregulation of metal efflux pumps, NADH quinone oxidoreductases, or other reactive oxygen species (ROS) scavenging enzymes upon exposure to U in the sulfate-reducing bacterium *Desulfotomaculum reducens* (15), *E. coli* grown at low pH (16), and the plant *Arabidopsis thaliana* (17). General and membrane stress responses were particularly pronounced when *Shewanella oneidensis* was exposed to U (18), and nucleic acid and protein damage have been shown to represent the primary modes of U toxicity in *Desulfovibio alaskensis* G20 (19). Phosphate transporters and cell wall proteins are known to be important for survival following U exposure for the budding yeast *Saccharomyces cerevisiae* (20), whereas thermoacidophilic archaea employ temporary degradation of cellular RNA as a dynamic mechanism for resisting U toxicity (21). However, there is still a significant

knowledge gap regarding the persistence of microbes in U-contaminated environments (9, 22), as the mechanisms for responding to acute U toxicity are not necessarily the same as those that enable survival and growth over a longer term.

We have chosen to focus on the aquatic organism *Caulobacter crescentus*, a widely-distributed nonpathogenic bacterium that can survive in low-nutrient environments with great potential to be exploited for the purpose of bioremediation (3, 23). *C. crescentus* is known to tolerate high levels of U(VI) and is able to facilitate U biomineralization through the formation of uranium phosphate precipitates (4). Transcriptomic and proteomic studies during U exposure in *C. crescentus* revealed dozens of genes with significant changes in expression in response to U; these genes do not appear to overlap substantially with those that are variably expressed in response to other heavy metal stresses, such as cadmium and chromium, suggesting a divergent cellular response to U(VI) (23, 24). This response manifests, in part, as a temporary arrest in cell cycle progression and DNA replication, along with some cell filamentation (a possible consequence of U-induced DNA damage) observed during growth recovery following U detoxification (25). Proteins upregulated in response to U include the periplasmic protein UrcA, a phytase enzyme, two-component signaling factors, and an ABC transporter. However, individual deletion of these upregulated genes did not increase U susceptibility (23, 24), indicating that these genes are not *required* for U tolerance. These results furthermore suggest that the cellular response to U is complex and includes more than a direct response to acute toxicity.

Here we use the Tn-seq approach to identify genes and gene products involved in U tolerance in *C. crescentus.* Compared to conventional transcriptomic and proteomic techniques, Tn-seq represents a novel tool for functional genomics that utilizes high-throughput, massively

parallel sequencing to uncover genes that contribute to cell fitness in a condition of interest (Fig. 1) (26, 27). The results presented here elucidate potential toxicity mechanisms of U and reveal novel genes and pathways important for growth and survival of *C. crescentus* in the presence of U.

MATERIALS AND METHODS:

Materials, bacterial strains, and growth conditions.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Peptone, yeast extract, and agar were purchased from Amresco (Solon, OH). Uranyl nitrate hexahydrate [$(UO_2)(NO_3)_2$•$6H_2O$] was obtained from SPI Supplies (West Chester, PA). A stock solution of uranyl nitrate (100 mM) was prepared in 0.1 N nitric acid. All PCR reactions were conducted using iProof polymerase from Bio-Rad (Hercules, CA) supplemented with 5% dimethyl sulfoxide (DMSO) according to manufacturer's instructions. *E. coli* WM3064 (Table S1) was maintained on solid LB medium supplemented with 300 µM diaminopimelic acid (DAP) and 1.5% agar. Liquid cultures were prepared in LB medium supplemented with 300 µM DAP. Where appropriate, LB was supplemented with kanamycin at 50 µg/ml for both liquid and solid media. *Caulobacter crescentus* NA1000 was maintained on solid PYE medium (0.2% peptone, 0.1% yeast extract, 0.5 mM $MgSO_4$, and 1 mM $CaCl_2$) supplemented with 1.5% agar (28). Liquid cultures were prepared in PYE medium. Buffered-PYE medium with 50 mM MES (pH 6.1) added was used for U spike-in experiments. Where applicable, PYE was supplemented with antibiotics at the following concentrations (for liquid/solid media, in µg/ml): kanamycin (5/20), spectinomycin (25/100), streptomycin (5/5), chloramphenicol (1/1), and tetracycline (1/2).

Construction of Tn-seq library and growth under U and Cd.

To construct the master library of *Tn5* mutants, the transposon delivery plasmid pXMCS2::*Tn5Pxyl* (29) was conjugated from an *E. coli* WM3064 donor strain into a *C. crescentus* Δ*recA* strain. Transposition events were selected on PYE-agar plates containing kanamycin (which selects specifically for mutants that carry the *Tn5Pxyl* transposon) and 0.1% xylose (which circumvents polar effects by allowing expression of genes downstream of the transposon from the *C. crescentus xylX* promoter). We note that genes downstream from the transposon are no longer under the control of their native promoter and are constitutively expressed from the *xylX* promoter. Plates were incubated at 30 ºC for 7 days. Two master libraries, each containing ~1.5 x 10^6 *Tn5Pxyl* insertion mutants, were prepared. Sets of ~10,000 colonies were pooled, re-suspended in PYE medium containing 10% glycerol, and stored at -80 ºC for subsequent selection on U and Cd plates.

For U or Cd selection, the frozen glycerol stocks were diluted in PYE medium and plated on PYE-agar plates containing 0.1% xylose and supplemented with either 250 μM uranyl nitrate (U library 1), 275 μM uranyl nitrate (U libraries 2 and 3), 12 μM cadmium sulfate, or no metal. Each stock was plated on enough plates to generate ~15,000 colonies with ~300-500 colonies on each plate. The PYE-agar plates were incubated at 30 ºC for 7 days. Each library contained ~1.5 x 10^6 *Tn*5 mutants, except U libraries 2 and 3, which had ~2.5 x 10^6 and ~5 x 10^5 *Tn5* mutants, respectively. Colonies were pooled and stored as described above.

DNA processing for sequencing.

From each library, 10 µl of each frozen glycerol aliquot was pooled and cells were harvested by centrifugation at 10,000 x *g* for 10 min. Genomic DNA was extracted from the cells using phenol-chloroform extraction according to standard procedures (30). One to three replicate DNA extractions were performed for each library (master, U, Cd, and no metal) and were processed and sequenced as described below.

One microgram of genomic DNA (gDNA) was sheared in a 120 µl volume using a Covaris E210 machine (Woburn, MA) and a Covaris AFA microTUBE tube. Each sample was sheared for 150 s with the following conditions: 10% duty cycle, intensity 5, and 200 cycles per burst. Sheared samples were then cleaned and size-selected with a double Agencourt Ampure XP bead cleanup (Beckman-Coulter, Brea, CA). The first round of the cleanup used a 0.85:1 volume ratio of beads to sample; the second round used a 1.4:1 volume ratio. One microliter of the size-selected gDNA was run on an Agilent Bioanalyzer DNA 1000 chip (Santa Clara, CA) to confirm a mean fragment size distribution of 200-250 base pairs. Each sample was then treated with end repair and A-tailing enzymes as described in the NEBNext DNA Library Prep for Illumina sequencing (New England Biolabs, Ipswich, MA) protocol. Following cleanup after A-tailing reactions, modified Illumina paired-end adapters were ligated to the DNA fragments. After adapter ligation, excess adapter and adapter dimer were removed with a 1:1 volume ratio of the Ampure XP beads. Samples were then amplified with Tn-specific and indexed primers (Table S2) using JumpStart Taq DNA polymerase (Sigma-Aldrich) in a 100 µl volume. This step selected for and amplified DNA fragments containing transposon insertion sites. Thermal cycling conditions were as follows: 94 °C for 2 min, [94 °C for 30 s, 65 °C for 20 s, 72 °C for 30 s] x 30, 72 °C for 10 min, 4 °C hold. PCR reactions were cleaned a final time with a 0.9:1 volume ratio of the Ampure XP beads and eluted in 25 µl of TE buffer (10 mM Tris, 1 mM

EDTA, pH 8.0). One microliter of each sample was run on an Agilent Bioanalyzer DNA 1000 chip to confirm the presence of a library and the absence of adapter/primer dimer. When excess dimer was observed, an additional 0.85:1 volume ratio of Ampure XP bead cleanup was performed with Agilent chip QC repeated.

DNA sequencing and mapping to the genome.

DNA was sequenced on an Illumina HiSeq 2000 (San Diego, CA, 150 bp read length, paired-end). Reads from desired transposon insertions into the *C. crescentus* genome have an expected sequence composition comprising of 1) 5 'random' nucleotides, 2) 35 bp corresponding to the terminal inverted repeat of the *Tn5* transposon, 3) 110 bp derived from the *C. crescentus* genome and/or read-through into the Illumina sequencing adapter. Reads beginning with the transposon *Tn5* sequence were identified using Cutadapt (31). *Tn5* sequence and any detectable sequences derived from Illumina sequencing adapters were removed using Cutadapt, leaving DNA corresponding to insertion sites in the *C. crescentus* genome. Trimmed sequences were subject to quality checking using Trimmomatic (32) to remove trailing sequences with a sliding window average quality score of 15 or less. Trimmed sequences were then aligned to the *Caulobacter crescentus* NA1000 reference genome sequence (NC_011916.1) using BWA (33). Bam alignment files were converted to BedGraph (reads vs. genome position) for viewing using custom Perl scripts. Transposon insertion sites were identified as the mapped location of the first base position of sequencing strings after trimming of the *Tn5* end sequences. Statistics from the sequencing analysis are summarized in Table 1.

Data analysis for U specific essentiality.

187 The open-source, web-based software ESSENTIALS (34) was used to analyze and
188 compare data from the test libraries (U, Cd, no metal) to the control master libraries. For each
189 pairwise analysis, BedGraph files for replicates originating from a single test library of mutants
190 were compared to BedGraph files from the control master libraries. The BedGraph files were
191 analyzed using the genome of *Caulobacter crescentus* NA1000 (NC_011916.1) with default
192 parameters, including use of "truncated.ppt" for truncation of the 3' end of genes, use of Loess
193 genomic position bias removal, TMM read count normalization, unpaired (qCML) analysis,
194 tagwise dispersion modeling of variance with a value of 5 for prior.n smoothing, and BH p-value
195 adjustment. Library sizes were inputted as 1,500,000. Normalized reads/gene, logFC ($\log_2$ fold
196 change in normalized total insertions per gene compared to the master libraries), and adjusted p-
197 values were outputted from the software (Table S3).
198 For heat map analysis, the raw counts of total insertions per gene were normalized using
199 the ESSENTIALS program with the master libraries as the reference data sets and all the test
200 libraries as the test sets. The heat map was generated using the default setting in the R heatmap
201 function for unsupervised hierarchical clustering.
202 In order to identify genes that have lower fitness under each test condition compared to
203 the master libraries, one needs to assign significant logFC and p-value cut-offs. Based on known
204 genes that are required for Cd survival (35) (Table S4), we used the Cd data set to determine the
205 logFC (< -0.9) and p-value (< 0.05) cut-offs. Genes previously identified as high-fitness or
206 essential in the genome of *C. crescentus* (29) were removed from further analysis due to
207 potential complications in analyzing whether U susceptibility for mutants in these genes is due to
208 poor fitness in general or a U-specific defect.

209 Genes with significantly lower fitness in U library 2, but not in the no metal or Cd
210 libraries were identified as U specific gene candidates (Table 2, Table S5). U library 2 was used
211 as the primary library because it had the most number of replicates and the most reproducible
212 data among replicates (data not shown). The U specific gene candidates identified based on U
213 library 2 were further divided into three regimes based on their degree of prevalence in the other
214 two U libraries: the top regime comprised genes that had significantly lower fitness in all 3 U
215 mutant libraries, while the middle and bottom regimes had genes that had significantly lower
216 fitness in 2 of the U libraries and in only 1 library (U library 2), respectively.
217
218 *Construction of deletion mutants.*
219 For construction of clean deletion mutants, ~500 bp regions upstream and downstream of
220 a given gene were PCR amplified using primers listed in Table S2. A three-way Gibson
221 assembly (Clontech In-Fusion HD Cloning Plus kit, Mountain View, CA) of the upstream and
222 downstream fragments into *Hind*III and *Eco*RI sites of pNPTS138 generated the plasmids listed
223 in Table S1. The sequences of the plasmids were confirmed by DNA sequencing. In-frame
224 deletion of a gene was generated using the respective plasmid through a standard homologous
225 recombination method as previously described (36, 37).
226 For construction of mutants lacking *rsaA*, a Tet^R cassette was amplified from pKO3 (38)
227 using *rsaAtet_for* and *rsaAtet_rev* primers (Table S2) and inserted into the *Nde*I site of pMCY04
228 (between the upstream and downstream flanking regions of *rsaA*) using Gibson assembly to
229 generate pMCY04a. In-frame replacement of *rsaA* with the Tet^R cassette was generated using
230 pMCY04a as previously described (38). This method was used because of difficulties in

obtaining clean deletions of *rsaA* under $rsaF_a$ and $rsaF_b$ mutant backgrounds due to unknown reasons.

U survival assay.

Strains of *C. crescentus* were pre-cultured in 2 ml of PYE supplemented with appropriate antibiotic(s) from single colonies at 30 ºC overnight. Cells were then diluted to an initial OD_{600} of 0.2 in PYE medium and cultured until OD_{600} reached ~0.4-0.6 (late exponential phase). Cells were harvested, washed, and resuspended in PIPES buffer (50 mM PIPES, pH 7.0) to a final OD_{600} of 0.5. Uranyl nitrate was added to the suspensions to a final concentration of 50 µM and incubated at room temperature (RT). Negative control without U was also included. After 1 h of U exposure, aliquots were removed, serial dilutions of 10^1 to 10^6 of the aliquots were prepared, and 5 µL of each dilution were spotted onto PYE-agar. After 2 days of incubation at 30 ºC, colony forming units (CFU) were determined. Fold of cell death was calculated by dividing the CFU/ml from assays without U by that with U. At least 4 biological replicates were included for each assay.

Growth in buffered PYE with U spike-in.

C. crescentus cells were pre-cultured from single colonies in 500 µL of PYE supplemented with appropriate antibiotic(s) at 30 ºC for 8 h. Cells were then diluted to an initial OD_{600} of 0.001 in PYE with appropriate antibiotic(s) and cultured for 16 h until the OD_{600} was about 0.6 (late exponential phase). Cells were harvested, spent medium was removed, and cells were inoculated into PYE medium supplemented with a final concentration of 50 mM MES pH 6.1 to an initial OD_{600} of 0.02. MES buffer was added to maintain pH; without buffering, the

medium pH would decrease upon addition of uranyl nitrate due to U hydrolysis and the nitric acid present in the U stock solution. Cells were cultured at 30 ºC, 220 rpm until OD_{600} reached 0.06 (early exponential phase), at which point uranyl nitrate was added to a final concentration of 350 µM. Controls without U addition were included. Cell density was monitored by OD_{600} and biological triplicates were included. Given that no significant cell elongation was observed during growth of *C. crescentus* in buffered-PYE containing U (data not shown), doubling times were estimated based on OD_{600} values.

Gene expression by Nanostring.

C. crescentus NA1000 and $rsaF_a$ mutant cells were grown in MES-buffered PYE medium as described above with U spiked in at early exponential phase. After 30 min of U exposure, 10 ml of cells was removed and transcription was immediately stopped by addition of 1.25 ml of a solution of 5% phenol/95% ethanol on ice. Cells were harvested by centrifugation at 4 ºC and total RNA was extracted from cells using TRIzol (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Samples were prepared in biological triplicates. Nanostring analysis to determine $\log_2$ expression levels was conducted as described elsewhere (39). Nanostring probes used for mRNA detection are listed in Table S6.

Cellular accumulation of U.

Cells were grown, washed, and re-suspended in PIPES buffer as described for the U survival assay above. Uranyl nitrate was added to the suspensions to a final concentration of 100 µM and incubated at RT for 30 min. Cell suspension was centrifuged at 20,000 x *g* for 5 min and the supernatant was collected and analyzed for U content using the ArsenazoIII colorimetric

277 assay as described previously (4, 40). An abiotic control was also performed without addition of
278 cells to control for abiotic U precipitation. All assays were performed in biological triplicates.
279 The amount of U accumulated by the cells was calculated as follows:
280 (U accumulation) = (total U) – (abiotic U precipitate) – (U in supernatant)
281
282 *Determination of antimicrobial sensitivity by agar disk diffusion assays.*
283 *C. crescentus* NA1000, *rsaF*$_a$ mutant, *rsaF*$_b$ mutant, and *rsaF*$_a$*F*$_b$ double mutant cells
284 were grown in PYE medium to exponential phase. Cultures were normalized and adjusted to
285 OD_{600} of 0.3 and 100 µl was plated on PYE-agar. Filter paper disks with a diameter of 7 mm
286 were placed on the agar with the following compounds (volume, concentration) pipetted onto a
287 disk: tetracycline (5 µl, 50 µg/ml), cadmium sulfate (10 µl, 40 mM), uranyl nitrate (10 µl, 200
288 mM), SDS (5 µl, 5% (w/v)), or hydrogen peroxide (5 µl, 100 mM). Plates were incubated at 30
289 ^{o}C for 2 days. Pictures of the agar plates were taken using a ChemiDoc imager (Bio-Rad) and
290 diameters of zones of inhibition were measured using ImageJ (41).
291
292 *U biomineralization assay.*
293 The U biomineralization assay was conducted as previously described (4). Briefly, cells
294 grown in PYE to late exponential phase were harvested, washed once with 10 mM NaCl, and re-
295 suspended in PIPES buffer to a final OD_{600} of 0.5. Biomineralization assays were started by the
296 addition of glycerol-2-phosphate and uranyl nitrate to final concentrations of 5 mM and 500 µM,
297 respectively, and incubated at 30 ^{o}C. At given assay time points, aliquots were removed to
298 quantitate soluble U using the Arsenazo III colorimetric assay and to determine CFU.
299

300 **RESULTS:**

301 *Tn-seq screen for U sensitive mutants.*

302 In our Tn-seq screening scheme (Fig. 1), master libraries of *Tn5* mutants were first
303 generated by selection on PYE-kanamycin plates according to Christen et al. (29). These master
304 libraries were subsequently exposed to each of three conditions on PYE plates to generate the
305 experimental libraries: 1) uranyl nitrate (U) at 250 or 275 μM, 2) cadmium sulfate (Cd) at 12
306 μM, or 3) no metal. The concentrations of U and Cd were chosen as the maximum
307 concentrations in which the wild type *C. crescentus* strain did not exhibit a significant growth
308 defect on PYE-agar supplemented with the given metal (data not shown). *Tn5* mutant generation
309 and selection on U were performed in two steps due to observed incompatibility between U and
310 kanamycin selection, presumably due to chemical interactions that block antibiotic activity when
311 U and kanamycin are used concurrently (data not shown). The 'no metal' condition identifies
312 mutants that have lower fitness due to conditions endured during the 2nd plating step, such as
313 freeze-thaw. The Cd condition controls for genes that are involved in tolerance to heavy metal
314 stress in general rather than U-specific stress. Each of the libraries obtained in this study
315 contained ~10^6 colonies in order to obtain enough mutants to saturate the genome (29).

316 Illumina Hi-seq was used to determine the genomic distributions of Tn5 insertions for
317 each library (29). One to three replicates of genomic DNA from a single library were isolated
318 and sequenced to test the reproducibility of our method. Of the 4-10 million reads generated for
319 each replicate, 77-91% passed initial filtering, demonstrating that the reads from the Hi-seq were
320 of high quality (Table 1). However, only 11-64% of the total reads were mapped to the *C.*
321 *crescentus* genome; for reasons unclear to us, the majority of the remaining reads mapped to the
322 pXMCS2::*Tn5Pxyl* plasmid used for transposon mutagenesis. Less than 1% of reads were

unmapped, representing non-specific PCR products. Despite the low proportion of mapped reads, ~100,000 – 300,000 unique insertion sites (*i.e.*, one insertion for every 12-36 bps on average) are represented in each data set, which is comparable to the insertion frequencies reported in other Tn-seq studies (42-44). By plotting the distribution of unique insertions as a function of gene length for every *C. crescentus* ORF, we were able to determine whether transposon insertions that are underrepresented in each data set correspond to the set of essential or fitness-relevant genes previously identified for *C. crescentus* (29) and thereby evaluate the reliability of the transposon mutagenesis scheme. We found that 96 ± 4% of *C. crescentus* ORFs identified as essential in the Christen et al. Tn-seq screen (29) are found within the quartile of *C. crescentus* genes with the lowest proportion of unique insertions per unit gene length in each data set (Fig. S1). These results demonstrate the integrity of each data set and validate the set of genes previously found to contribute substantially to fitness or viability.

Using ESSENTIALS, an open-source, web-based algorithm (34), we compared and normalized data from each experimental library (U library 1, 2, 3, Cd, no metal) to data obtained from the master libraries in a pairwise fashion (test library vs. master libraries). This analysis enabled determination of insertion frequency (transposon insertions/gene) for each gene and the fold change in insertion frequency for every gene in each experimental condition relative to the master libraries. Heat map representation of all normalized data sets revealed that replicates from the same experimental library clustered together, showing high reproducibility among biological replicates (Fig. S2). The 3 U libraries were only loosely clustered, which is likely attributed to the differences in U concentration (250 μM used in U library 1 compared to 275 μM in U libraries 2 and 3) and mutant library size (0.5×10^6 mutants in U library 3 compared to $1.5 – 2.5 \times 10^6$ mutants in U libraries 1 and 2).

346 We define U-specific fitness genes as those ORFs that exclusively exhibit lower insertion
347 frequencies in the U libraries compared to the master libraries (Fig. 2). The Cd data set was used
348 to assign cut-offs for logFC (< -0.9) and p-values (< 0.05) representing lower insertion frequency
349 based on genes that are known to be required for fitness in the presence of Cd (35) (Table S4).
350 Using these criteria, we identified 37 U-specific candidates (Table S5). We note that these
351 candidates are not those with the largest differences in insertion frequency between the master
352 and U libraries; the more extreme outliers were eliminated because they were also extreme
353 outliers in the Cd and no metal data sets and likely represent a category of genes that affect
354 survival during the second plating step. From the list of 37 U-specific candidates, we selected 15
355 candidate genes for further study with priority toward those genes identified in all 3 U libraries
356 (Table 2). Mutants of each of the 15 genes were obtained (Table S1) and first tested for their
357 ability to grow in the presence of PYE supplemented with 300 μM uranyl nitrate without pH
358 buffering (Fig. S3), conditions similar to those used in the Tn-seq screen. Results revealed that
359 10 of the 15 mutants exhibited a growth defect in the presence of U relative to wild type. We
360 later discovered that the pH of PYE decreases from 6.5 to 5.7 with addition of 350 uM uranyl
361 nitrate, affecting growth phenotypes (25). To uncouple U response from changes in pH and
362 growth conditions, we tested the ability of each mutant to survive U exposure under buffered
363 conditions (Fig. 3), and found that 4 of the 15 mutants showed significantly higher U
364 susceptibility than wild type. These four mutants will be discussed in the following sections.
365
366 *$rsaF_a$ and $rsaF_b$ are important for U fitness.*
367 Of the candidates tested, *$rsaF_a$* and *$rsaF_b$* mutants were observed to have the most
368 significant reduction in CFU/ml in the U survival assay, with a ~10 fold lower survival rate

compared to the wild type when exposed to U. While the *rsaF$_a$F$_b$* double mutant showed a similar reduction in survival rate as the single mutants, it exhibited a survival defect even in the absence of U, unlike the single mutants, suggesting that the double mutant had poor fitness in general (Fig. 3). *rsaF$_a$* and *rsaF$_b$* are two homologous genes encoding for the outer membrane proteins RsaF$_a$ and RsaF$_b$ that are part of the type I protein translocation pathway for exporting the highly abundant S-layer protein RsaA to the cell surface of *C. crescentus* (45). The fact that *rsaF$_a$* and *rsaF$_b$* are located 322 kb apart in the genome and that we were able to identify both genes in our Tn-seq screen suggests that they are individually critical for U tolerance. Furthermore, expression analysis revealed that *rsaF$_a$* and *rsaF$_b$* transcripts are upregulated ~4-fold and ~10-fold, respectively, during growth in buffered PYE by the addition of 350 μM U (Fig. 4), implying a concerted regulatory response for the RsaF transport system in U resistance.

To confirm that these survival defects are not the result of a compromised S-layer, we tested U susceptibility of *rsaA*, *rsaD*, and *rsaE* null mutants, which are deficient in various factors required for S-layer biogenesis (Fig. 3). RsaA is the structural protein that comprises the S-layer, and RsaD and RsaE are the ATP-binding ABC transporter and membrane fusion protein, respectively, that, together with RsaF, form the channel to export RsaA across the cell envelope (46); all mutants were previously shown to be deficient in S-layer production (45, 46). In contrast to the RsaF mutants, the *rsaA*, *rsaD*, and *rsaE* null mutants showed similar CFU/ml as compared to wild type in the presence of U, suggesting that 1) the S-layer *per se* does not confer U resistance and 2) RsaD and RsaE are not involved in U tolerance, indicating that the mechanism of U tolerance does not rely on components of the S-layer transport system other than RsaF.

In addition to cell survival, *rsaF* mutants were tested for U tolerance during growth in buffered PYE medium in a spike-in experiment, where U was added during early exponential phase (Fig. 5, Fig. S4). We observed that the *rsaF$_b$* mutant did not exhibit any significant change in doubling time with or without U, whereas the *rsaF$_a$* mutant exhibited an increase in doubling time compared to wild type whether or not U was added, indicating a general (U-independent) growth defect. Unexpectedly, the *rsaF$_a$F$_b$* double mutant reversed this defect; in the absence of U, the double mutant grew at a rate only slightly slower than the wild type. The double mutant still exhibited a significant increase in doubling time in U-containing medium compared to wild type, suggesting that RsaF$_a$, but not RsaF$_b$, is needed for U tolerance during growth. The unexpected lack of direct correlation between growth and survival phenotypes in the *rsaF* mutants (compare Figs. 3 & 5) indicates that mechanisms of U toxicity during active growth and the survival assay are different. The cause of the general growth defect observed for the *rsaF$_a$* mutant is unclear.

To determine whether the increased U sensitivity observed in mutants lacking one or both RsaF subunits is an indirect consequence of intracellular accumulation of RsaA, we prepared *rsaA* gene deletions in the *rsaF$_a$*, *rsaF$_b$* and *rsaF$_a$F$_b$* mutant backgrounds and measured the change, if any, in U tolerance in survival and growth assays. In the survival assay, all three of the deletion strains constructed (*rsaF$_a$rsaA*, *rsaF$_b$rsaA*, and *rsaF$_a$F$_b$rsaA*) showed survival rates in the presence of U similar to those of their respective *rsaA*$^+$ parents (~10^2- to 10^3-fold more cell death relative to the no-U control), suggesting that U sensitivity is not caused or exacerbated by RsaA accumulation (Fig. S5A). However, we did observe that the U-independent survival defect of the *rsaF$_a$F$_b$* double mutant (Fig. 3, Fig. S5A) was completely suppressed by the deletion of *rsaA*. This result demonstrates that intracellular RsaA accumulation contributes to

414 the general survival defect of the *rsaF$_a$F$_b$* mutant. In the growth assay, the *rsaF$_a$rsaA* mutant
415 displayed a prolonged doubling time relative to the *rsaF$_a$* single mutant regardless of U addition,
416 suggesting that the slow growth of this strain is not caused by cytoplasmic RsaA (Fig. S5B).
417 Interestingly, the *rsaF$_a$F$_b$ rsaA* triple mutant grew slightly better than the *rsaF$_a$F$_b$* mutant both in
418 the presence and in the absence of U (Fig. S5B), indicating that cytoplasmic RsaA accumulation
419 may indeed slow growth in cells lacking RsaF activity. We therefore used the *rsaF$_a$F$_b$rsaA* triple
420 mutant in place of the *rsaF$_a$F$_b$* double mutant in subsequent U uptake experiments, in order to
421 avoid complications in interpretation due to the effects of intracellular RsaA accumulation.
422 Finally, to confirm the results of our Tn-seq experiment, we examined the ability of the
423 *rsaF$_a$F$_b$rsaA* triple mutant to survive on solid PYE-agar containing either 300 μM uranyl nitrate,
424 12 μM cadmium sulfate, or no metal (Fig. S5C). While wild-type *C. crescentus* exhibited full
425 survival under all conditions tested, the *rsaF$_a$F$_b$rsaA* mutant exhibited a survival defect in the
426 presence of U (~10^2 fold cell death), but not Cd or the no metal control. In contrast, a deletion
427 mutant of the Cd efflux transporter *ccna_02807* exhibited a survival defect in the presence of Cd,
428 but not U or the no metal control. These results validated the results of the Tn-seq screen from
429 which mutants in the *rsaF* system were identified.
430
431 *RsaF resembles TolC from E. coli.*
432 Smit and coworkers have previously reported that the two RsaF proteins are both
433 homologues of TolC, a multifunctional *E. coli* outer membrane protein known to interact with
434 several efflux pump partners in transporting a variety of compounds (including antibiotics and
435 metals) from the cytoplasm to outside the cell (45). A BLAST search of the *C. crescentus*
436 genome indicates that RsaF$_a$ and RsaF$_b$ have the most significant identity to TolC with sequence

437 identities of 23% and 26% (87% coverage), respectively (47). Given the evolutionary
438 relationship with the TolC efflux pump, we hypothesized that $RsaF_a$ and $RsaF_b$ may confer U
439 tolerance by negotiating the export of U through an RsaF-dependent pathway that can be
440 genetically separated from S-layer biogenesis.

441 To test this hypothesis, we quantified U accumulation in wild type and the *rsaF* mutants
442 (Fig. 6). We reasoned that higher levels of U accumulation should be observed in the *rsaF*
443 mutants (relative to wild type) if the TolC-like RsaF proteins play a role in U efflux. Consistent
444 with this prediction, the $rsaF_a$ and $rsaF_aF_brsaA$ mutants exhibited ~30% and ~20% higher U
445 accumulation, respectively, than the wild type after 30-min of U exposure. The increase in U
446 accumulation in these strains cannot be attributed to S-layer perturbation, since the *rsaA* single
447 mutant exhibited the same amount of U accumulation as the wild type. Since the $rsaF_b$ mutant
448 did not exhibit increased U accumulation, the observations together suggest that $RsaF_a$ may play
449 a specific role in the prevention of U accumulation in *C. crescentus*. However, we cannot rule
450 out the possibility that the higher U accumulation observed in the $rsaF_a$ and $rsaF_aF_brsaA$
451 mutants could be due to defects in outer membrane integrity. Attempts to measure U efflux were
452 not successful (data not shown), which may be due to potential U precipitation or cell wall
453 adsorption.

454 To determine whether the *C. crescentus rsaF* system could behave similarly to a TolC-
455 like efflux system, the susceptibility of the *rsaF* mutant strains against several antimicrobial
456 substrates of TolC-utilizing type I secretion systems (48) was determined using a disk diffusion
457 antibiotic sensitivity assay (Fig. 7). All three *rsaF* mutants showed larger zones of growth
458 inhibition in the presence of tetracycline or U compared to the wild type strain. Mutants of $rsaF_a$
459 and $rsaF_aF_b$, but not $rsaF_b$ alone showed larger inhibition zones in the presence of Cd or SDS.

460 Thus, $RsaF_a$ appears to be the primary factor involved in resistance to these two compounds. We
461 note that Cd sensitivity was only observed in the disk diffusion assay, but not under the
462 conditions of the Tn-seq screen (Fig. S5C), which is likely due to higher Cd concentrations in the
463 disk diffusion assay, resulting in increased sensitivity. Together, these results demonstrate that
464 *rsaF* mutants show broad antimicrobial susceptibility that is not limited to U, similar to TolC in
465 *E. coli.* The *rsaF* mutants were also tested for susceptibility to chloramphenicol, ethidium
466 bromide, zinc, and chromium and exhibited the same degree of susceptibility as wild type (data
467 not shown). The mechanisms of antimicrobial selectivity by the *rsaF* system, however, are
468 currently unclear.

469 Previous reports indicate that the lack of TolC can induce oxidative stress in *E. coli* (49).
470 Reasoning that this confounding effect could at least partially account for the apparent U
471 sensitivity of *rsaF* mutants, we tested whether the deletion of $rsaF_a$ leads to an increase in
472 oxidative stress in *C. crescentus*. To do this, we measured the transcription of oxidative stress
473 genes in wild type and the $rsaF_a$ mutant grown in buffered PYE with and without U (Fig. S6A).
474 Results indicated that the expression levels of key oxidative stress genes (*sodB, katG, oxyR, dps*)
475 are not significantly changed in the $rsaF_a$ mutant, suggesting that deletion of $rsaF_a$ is not
476 sufficient to induce an oxidative stress response and that U toxicity is not an indirect effect of
477 increased oxidative stress in the $rsaF_a$ mutant. Consistently, all *rsaF* mutants tested showed
478 similar susceptibility to hydrogen peroxide stress compared to the wild type in the disk diffusion
479 assay (Fig. 7). In addition, we note that genes involved in heat shock, DNA damage, and outer
480 membrane stress (*e.g.,* LpxC) (Fig. S6B, C, and D, respectively) also exhibited similar
481 expression levels between wild type and the $rsaF_a$ mutant, indicating that the loss of $RsaF_a$ does
482 not cause activation of common cytoplasmic or envelope stress responses. Significantly, the

483 expression of LpxC, which is involved in LPS biosynthesis (50), is also unchanged, arguing
484 against significant remodeling of the LPS. However, we cannot conclusively rule out the
485 possibility that the lack of *rsaF*$_a$ itself in some way affects the composition and/or properties of
486 the outer membrane.
487
488 *CztR-cztA*.
489 The *cztR* and *cztA* genes were also significant hits in our Tn-seq screen. The *cztR* gene
490 encodes a LysR family transcriptional regulator that, along with the putative CztA transporter,
491 has been implicated in response to Zn limitation and Cd exposure (51). To characterize the
492 contribution of CztRA to U tolerance, we performed growth and survival assays in strains
493 lacking *cztR* or *cztA*. Mutant lacking *cztR* exhibited lower fitness upon U exposure, with ~10
494 fold increase in cell death compared to wild type (Fig. 3). Consistently, the *cztR* mutant
495 exhibited a U-dependent growth defect, with a significant increase in doubling time in the
496 presence of U compared to wild type (Fig. 5, Fig. S4). Conversely, no discernable changes in
497 cell survival or U sensitivity in growth were apparent following deletion of *cztA* (Figs. 3 & 5,
498 Fig. S4). There was no significant change in *cztR* expression upon U exposure (Fig. 4). An ~8-
499 fold increase in *cztA* expression occurs in the presence of uranyl nitrate; however, this increase
500 may be due to nitrate present, since an ~8-fold increase in *cztA* expression also occurred in the
501 presence of potassium nitrate (data not shown). Although the CztRA system may not be
502 specifically induced by U, it clearly plays a role in U fitness both under survival and growth
503 conditions. Given its previously reported function in Cd resistance (35, 51), the CztRA system
504 may be involved in general heavy metal stress response.
505

506 *spoT*.

507 A mutant lacking *spoT* was observed to have a lower fitness with U, with an additional
508 ~10-fold reduction in CFU/ml upon U exposure compared to wild type (Fig. 3). The *spoT* gene
509 encodes a ppGpp hydrolase/synthetase that was previously shown to be involved in the stringent
510 response in many bacteria including *C. crescentus* and *E. coli* (52, 53). Deletion of *spoT* in *C.*
511 *crescentus* resulted in cell death upon carbon starvation due to unconstrained chromosome
512 replication (54). In order to test whether the cell death observed with the *spoT* mutant upon U
513 exposure is related to carbon starvation, we repeated the U survival assay in the presence of
514 glucose (Fig. 8). The *spoT* mutant showed a similar survival rate as wild type upon U exposure
515 in the presence of glucose, suggesting that the observed U defect in the absence of glucose is a
516 consequence of carbon starvation rather than U-specific toxicity. We note that U toxicity is
517 somewhat mitigated by the presence of glucose, presumably due to complexation with U.
518 Consistently, the growth assay indicated that the doubling time of the *spoT* mutant is similar to
519 wild type regardless of the presence of U (Fig. 5), and in the wild type strain, the expression
520 level of *spoT* remained unaltered by U (Fig. 4). Thus, *spoT* appears to be important for U
521 tolerance only under carbon starvation conditions, likely resulting from enhanced U-toxicity to
522 cells with unchecked DNA replication (54).

523

524 *Biomineralization activity of mutants.*

525 We have previously found that *C. crescentus* actively biomineralizes U via its native
526 phosphatase activity and that perturbed biomineralization activity causes survival defects during
527 U exposure (4). To test whether the U-sensitive mutants identified above retain their U
528 biomineralization activity, we performed the U biomineralization assay using glycerol-2-

phosphate as the phosphate source for the *rsaF*, *cztR*, *cztA*, and *spoT* mutants (Fig. 9A). The Δ*phoY* strain, which lacks biomineralization activity and exhibits a severe survival defect in the presence of U (4), was included as a negative control. All mutant strains tested had similar activity to wild type with more than 85% of the U mineralized after 6 h, demonstrating that none of the strains had perturbed U biomineralization activity. Cell survival analysis under the U biomineralization condition (Fig. 9B) showed that all strains, with the exception of the *cztA* mutant, had a ~10 fold reduction in CFU/ml compared to wild type, indicating that U sensitivity in these strains is independent of U biomineralization. These cell survival trends are similar to those observed under non-mineralizing conditions (Fig. 3). We note that the effective U toxicity under mineralizing conditions is much lower than that under non-mineralizing conditions, presumably due to U complexation with glycerol-2-phosphate (4). Cell death exhibited by the Δ*phoY* control strain was highest (~100 fold), showing that lack of biomineralization activity caused the most severe susceptibility to U under mineralizing conditions.

DISCUSSION:

Tn-seq is a powerful whole-genome genotypic screening method that has recently been widely employed to identify genes that are critical for survival upon exposure to antibiotics and various other chemical stresses (27, 42, 55). We have performed Tn-seq and identified transposon insertions in 37 distinct genes that result in reduced fitness under U stress, 15 of which we chose for further study. Individual single mutants lacking each of these 15 genes were tested for U susceptibility, and 10 of these mutants were found to exhibit a growth defect in un-buffered PYE supplemented with U, with only 4 mutants confirmed to have a U survival defect in a buffered solution. Besides the potential difference in U susceptibility under different testing

552 conditions, the low correlation between survival phenotype and Tn-seq fitness could also be due
553 to the low stringency used in selecting candidate genes from our screen. Almost all of the genes
554 that we identified had logFC values between 1 and 2 compared to the master libraries,
555 corresponding to 2- to 4-fold reductions in fitness. These values are significantly lower than
556 values found in other Tn-seq studies examining antibiotic resistance and other environmental
557 stresses (27, 42, 55). Gallagher et al. reported that genes with weak negative selection of 2.5- to
558 5-fold reduction have a low correlation between phenotype and Tn-seq fitness (42). Given that
559 similar numbers of unique insertions are present in our data sets compared to other Tn-seq
560 studies, we believe that the low fitness values are indicative of the fact that *C. crescentus* has a
561 broad and relatively graded response to U toxicity. Consistent with this notion, our previous
562 proteomic and transcriptomic studies (23, 24) also indicate that a small group of specialized
563 genes cannot account for U resistance. Instead, the response occurs on a global level, involving
564 many genes from different pathways serving functions that may act redundantly to mitigate U
565 toxicity. These results highlight the need to conduct detailed phenotypic analysis of mutants
566 following Tn-seq screening.

567 Despite the weak negative selection by U, the Tn-seq screen was successful in identifying
568 outer membrane transporters $RsaF_a$ and $RsaF_b$, homologues of TolC in *E. coli*, as factors
569 important for U resistance. Deletion of $rsaF_a$ resulted in higher U susceptibility as well as higher
570 levels of U accumulation. In contrast, the $rsaF_b$ mutant did not show a significant U growth
571 defect or higher U accumulation. We also observed that the single $rsaF_a$ deletion had higher U
572 susceptibility and accumulation of U as compared to the $rsaF_aF_b$ double mutant. The reason for
573 these results is unclear, but may reflect differential gene expression when $rsaF_a$ alone is
574 disrupted versus both genes, leading to different pleotropic effects. Although $RsaF_a$ and $RsaF_b$

appear to have redundant functions in S-layer export in *C. crescentus* (45), our results suggest that $RsaF_a$ plays a dominant role in U resistance. Like TolC, RsaF likely exists exclusively as a trimer in the OM, although it is currently unclear whether it preferentially forms homotrimers or heterotrimers consisting of both $RsaF_a$ and $RsaF_b$. Our ongoing efforts focus on building functional RsaF structures computationally and experimentally and testing the preference of different RsaF models/compositions toward drugs/cations.

Given the results from our study, we speculate that RsaF behaves like TolC and acts as an outer membrane efflux pump to enable excretion of a variety of antimicrobials and metals, including U. The increased susceptibility of *rsaF* mutants to tetracycline, cadmium, and SDS, compounds known to be exported via type I secretion systems (48, 56, 57), suggests that RsaF may be involved in active export of these antimicrobial compounds via a mechanism similar to TolC (48). TolC proteins are known to interact with various inner membrane translocases/pumps, consisting of an adaptor protein and an energy-providing protein, typically an ATP-binding cassette protein or proton antiporter of the RND or MFS superfamily (58), which provide specificity to export particular substrates. In *E. coli*, TolC is multifunctional and known to interact with several translocases/pumps, including HlyBD for hemolysin toxin secretion and AcrAB for multidrug efflux (58). In *C. crescentus*, Smit and coworkers previously identified $RsaF_a$ and $RsaF_b$ as the TolC-like proteins responsible for export of the S-layer protein RsaA and recently the S-layer associated metalloprotease SapA via the translocase comprised of RsaDE (45, 46, 59). U is unlikely to use the same export system as the S-layer protein, however, given that deletion of RsaD and RsaE do not exhibit increased U susceptibility. Instead, we hypothesize that RsaF may interact with other translocases/pumps to export compounds other than protein, such as antibiotics and metals. There are several annotated type I

598 pumps for multidrug (*ccna_01261*, *ccna_01865*, *ccna_03218*) and metal (*ncc/czr* and *ncz*
599 systems for Ni/Cd/Co/Zn) export in the *C. crescentus* genome for which the outer membrane
600 export partner is unknown (57, 60). In addition, our Tn-seq screen identified a putative
601 multidrug resistance protein B (CCNA_03831) as one of the targets with low U fitness (Table
602 S5). These proteins serve as potential inner membrane module candidates that interface with
603 RsaF to actively export antibiotics and metals via TolC-like systems, although further work is
604 needed to determine if they interact with RsaF and have export activities.

605 Alternatively, RsaF may play a role in maintaining the integrity and function of the outer
606 membrane that acts as a protective layer against antimicrobial compounds. Thus, the absence of
607 RsaF could result in outer membrane perturbation and leakage, leading to increased U
608 permeability, accumulation, and susceptibility. A defective outer membrane could also cause
609 increased sensitivity to antibiotics if a defective outer membrane no longer acts as a barrier to the
610 antibiotics. It is well known that outer membrane integrity affects susceptibility of microbes to
611 antimicrobial compounds (61), especially those with hydrophobic properties. The fact that the
612 $rsaF_a$ mutant is sensitive to the detergent SDS is a strong indication that outer membrane barrier
613 function is perturbed in these mutants, directly or indirectly caused by RsaF disruption. Studies
614 in *E. coli* have also shown that TolC mutants have a variety of pleotropic effects including
615 oxidative stress (49, 62, 63), peptidoglycan biosynthesis (64), regulation of outer membrane
616 composition (62, 63, 65), and cell morphology under Fe limitation (66). While it is feasible that
617 these pleotropic effects occur in RsaF mutants, we note that genes involved in oxidative stress or
618 membrane stress were not significantly differentially regulated in the $rsaF_a$ mutant compared to
619 wild type, although our analysis was limited to a small subset of genes for this experiment.

620 Finally, another promising set of genes that we identified in our Tn-seq screen are *cztR*
621 and *cztA*, which are annotated as a LysR family regulator and sodium bicarbonate transporter,
622 respectively. Marques and coworkers found that CztR auto-regulates its own expression and
623 positively regulates expression of CztA, demonstrating a link between these two proteins (51).
624 They propose that the CztRA system is involved in zinc transport under zinc-limited conditions
625 and that CztR may be involved in regulation of other oxidative stress genes (51). We found that
626 a *cztR* mutant, but not a *cztA* mutant, is more susceptible to U both in growth and survival,
627 suggesting that CztR does not confer U resistance by activating expression of *cztA*. We suspect
628 that the identification of the *cztA* gene in the Tn-seq screen is an artifactual consequence of the
629 organization of *cztA* and *cztR* into a single operon. As a transcriptional regulator, CztR may
630 facilitate U resistance by activating genes that are required for U detoxification, including
631 oxidative stress genes which have been demonstrated to be upregulated in response to U (23)(D.
632 M. Park, unpublished data). Interestingly, these two genes were also previously identified in a
633 transposon mutagenesis screen of *C. crescentus* under cadmium stress (35). Supporting these
634 prior results, we found that *cztR* and *cztA* also exhibited significantly lower fitness under Cd
635 selection in our Tn-seq screen (Table 2), suggesting that the system may be involved in general
636 heavy metal stress response. The magnitude of logFC values for these genes in the U libraries,
637 however, were even higher than in the Cd libraries, indicating that CztR may be more important
638 for growth in U compared to Cd.
639 In summary, our Tn-seq screen for genes involved in detoxification and stress regulation
640 during U exposure by *C. crescentus* has yielded several novel targets whose identity and function
641 had previously remained elusive through other types of omics analyses (23, 24). The results
642 highlight that genes necessary for longer term growth and survival under U stress do not

643 significantly overlap with those that are upregulated in response to acute U stress. Our results
644 thus demonstrate the power of using Tn-seq to conduct genotypic profiling to identify novel
645 genes required for fitness under stress conditions. These novel genes are crucial leads to a
646 detailed mechanistic understanding as to how bacteria are able to resist U in the environment and
647 thus have potential utility in informing future bioremediation strategies.
648
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848 **FIGURE LEGENDS:**

849

850 **Fig. 1. Scheme for our Tn-seq method.** A hypersaturated *Tn5* mutant library was first
851 generated by kanamycin selection (round 1), after which the library was exposed to U, Cd, or no
852 metal selective pressure (round 2). Genomic DNA was then extracted, sheared by sonication,
853 and Illumina adaptors were added to the sheared fragments. Genomic regions adjacent to the
854 *Tn5* insertions were PCR amplified using an indexed primer and a *Tn5* specific primer. PCR
855 fragments were subsequently sequenced by Illumina paired-end sequencing. The *Tn* insertion
856 site was determined for each mutant and the insertion frequency for each gene was calculated.
857 Genes important for U fitness are expected to have a lower insertion frequency under the U
858 condition compared to the no metal or Cd control conditions.

859

860 **Fig. 2. Comparison of insertion frequency among mutants under different selective**
861 **pressure.** (A-C) Plots of log(total insertions/gene length) for U library 2 (A), no metal (B), or
862 Cd (C) condition versus the master libraries prior to selection. Genes with lower fitness under U
863 (*rsaF*$_a$, *rsaF*$_b$, *cztR, cztA, spoT*) are highlighted with red circles (●). Genes with lower fitness
864 under Cd (*ccna*_02805-02811, Ni/Co/Cd resistance cassette) are highlighted with orange
865 triangles (▲). Linear regression analyses of the data are shown as blue lines. (D-G) Total
866 insertion counts at representative genome loci with lower U fitness are compared in the master
867 libraries (black), U library 2 (red), no metal library (blue), and Cd library (orange). Regions
868 containing the following genes are shown: (D) *rsaF*$_b$ (*ccna*_01379); (E) *rsaF*$_a$ (*ccna*_01067); (F)
869 *cztA* and *cztR* (*ccna*_03624-03625); and (G) *spoT* (*ccna*_01622). Plots were generated using
870 Mochiview (67), with insertion counts averaged among replicates from the same library.

871

872

873 **Fig. 3. Comparison of U survival by mutants identified by Tn-seq.** Strains were exposed to
874 no U control (black bars) or 50 μM uranyl nitrate (gray bars) in 50 mM PIPES buffer at pH 7.0
875 for 1 h after which CFU/ml were counted (left axis). Red bars represent the fold change in
876 CFU/ml between the U condition and the control (right axis). Error bars represent standard
877 deviations from at least 4 biological replicates. Mutants showing a lower U survival rate (*i.e.*,
878 higher susceptibility to U) compared to wild type are highlighted with asterisks (*p-value <
879 0.01).

880

881 **Fig. 4. Transcript levels of select gene candidates in response to U.** *C. crescentus* cells were
882 grown in buffered-PYE medium to early exponential phase and spiked without (black bars) or
883 with (gray bars) 350 μM uranyl nitrate. Samples were collected 30 min after spike-in and
884 transcript levels were determined by Nanostring analysis. Log_2 expression values are presented
885 and error bars represent standard deviations from 3 biological replicates. Transcripts
886 significantly up-regulated are highlighted with asterisks (*p-value < 0.01).

887

888 **Fig. 5. Comparison of doubling times of mutant strains in the presence or absence of U.**
889 Mutants were grown in buffered-PYE medium to early exponential phase and spiked without
890 (black bars) or with (gray bars) 350 μM uranyl nitrate. Doubling times were estimated based on
891 OD_{600} values during growth. Error bars represent standard deviations from 3 biological
892 replicates. Mutants showing a significant increase in doubling time compared to wild type under
893 the same conditions (-/+ U) are highlighted with asterisks (*p-value < 0.02, **p-value < 0.0001).

894

Fig. 6. Comparison of U accumulation by wild type and *rsaF* mutants. Amounts represent U accumulated by 8 x 10^7 cells after 1 h U exposure in 50 mM PIPES buffer at pH 7.0. Error bars represent standard deviations from 3 biological replicates. Mutants showing higher U accumulation compared to wild type are highlighted with asterisks (*p-value < 0.002).

Fig. 7. Comparison of sensitivity of wild type and *rsaF* mutants to different antimicrobials using an agar disk diffusion assay. Diameters of zones of inhibition are shown in mm. Tet, tetracycline; Cd, cadmium sulfate; U, uranyl nitrate; SDS, sodium dodecyl sulfate; H_2O_2, hydrogen peroxide. Wt, wild type, black bars; *rsaF*$_a$ mutant, gray bars; *rsaF*$_b$ mutant, striped bars; *rsaF*$_a$*F*$_b$ double mutant, crossed bars. Error bars represent standard deviations from 3 biological replicates. Mutants showing a significant increase in diameter compared to wild type are highlighted with asterisks (*p-value < 0.02, **p-value < 0.01).

Fig. 8. Comparison of cell survival of wild type and *spoT* mutant in response to U in the presence or absence of glucose. Strains were exposed to no U (black bars) or 50 μM uranyl nitrate (gray bars) supplemented with and without 1% glucose in 50 mM PIPES buffer at pH 7.0 for 1 h after which CFU/ml were counted (left axis). Red bars represent the fold change in CFU/ml between the U condition and the control (right axis). Error bars represent standard deviations from 4 biological replicates. Higher susceptibility to U compared to wild type is highlighted with an asterisk (*p-value < 0.01).

Fig. 9. Comparison of U biomineralization activity and cell survival of mutant strains under U mineralizing conditions. A) U biomineralization activity. Wild type (■), *phoY* mutant (●), *rsaF*$_a$ mutant (▲), *rsaF*$_b$ mutant (♦), *rsaF*$_a$*F*$_b$*rsaA* triple mutant (□), *cztR* mutant (○), *cztA* mutant (Δ), and *spoT* mutant (◊). Error bars represent standard deviations from 3 biological replicates. B) Cell survival under U mineralizing conditions after 6 h incubation. CFU/ml (left axis) at t=0 h (black bars) and t=6 h (gray bars). Fold change in CFU/ml between 0 and 6 h (red bars, right axis). Mutants showing significantly higher susceptibility to U compared to wild type are highlighted with asterisks (*p-value < 0.03; **p-value < 0.01).

TABLES:

Table 1. Statistics for Tn-seq analysis.

Library	Replicate[a]	Total reads	Reads (%) after filtering	Reads (%) mapped to genome	No. unique insertions	Insertion resolution (bp/insertion)
Master Tn library 1	1	4,622,651	3,879,040 (84)	1,128,991 (29)	254,837	14
Master Tn library 2	1	6,821,312	5,648,392 (83)	1,655,997 (29)	299,848	12
Uranium library 1[b]	1	5,258,684	4,475,230 (85)	1,407,024 (31)	172,227	21
	2	4,533,632	4,105,593 (91)	471,566 (11)	102,123	36
Uranium library 2[c]	1	4,345,587	3,535,408 (81)	1,570,567 (44)	229,517	16
	2	3,769,970	3,073,357 (82)	1,421,032 (46)	214,127	17
	3	5,484,725	4,452,924 (81)	2,078,630 (47)	233,053	16
Uranium library 3[c]	1	5,281,822	4,168,258 (79)	2,382,532 (57)	172,368	21
No metal library	1	10,718,904	9,297,151 (87)	1,160,988 (12)	150,231	25
	2	8,977,647	7,058,714 (79)	2,848,953 (40)	215,835	17
Cadmium library	1	4,771,097	3,685,040 (77)	2,342,281 (64)	213,944	17

[a] Replicates are different DNA isolations from the same Tn mutant library.
[b] Uranium library 1 was conducted at 250 µM uranyl nitrate.
[c] Uranium libraries 2 and 3 were conducted at 275 µM uranyl nitrate.

935 **Table 2. Select gene candidates with low U fitness.**

Gene	Annotation	Log$_2$FC compared to master libraries				
		Uranium library 1	Uranium library 2	Uranium library 3	No metal library	Cadmium library
CCNA_00080	LexA-like transcriptional repressor	-1.17*	-2.43**	-1.59**	-0.70	-0.31
CCNA_03858	*secB*, protein translocase subunit	-1.67**	-1.97**	-3.32**	-0.14	1.46**
CCNA_03624	*cztA*, sodium bicarbonate cotransporter	-1.79**	-1.54**	-2.58**	-0.14	-0.79*
CCNA_00285	acetylglutamate kinase	-0.97*	-1.50**	-2.00**	-0.87	0.55
CCNA_01379	*rsaF$_b$*, type I secretion outer membrane protein	-0.93*	-1.41**	-1.91**	-0.13	0.20
CCNA_03625	*cztR*, LysR family transcriptional regulator	-1.29**	-1.41**	-2.26**	-0.06	-0.70
CCNA_03498	MarR/EmrR family transcriptional regulator	-0.98*	-1.36**	-2.23**	-0.42	0.42
CCNA_01521	hypothetical protein	-0.98**	-1.20**	-3.29**	-0.69	-0.12
CCNA_00290	autotransporter protein	-0.98**	-1.04**	-1.74**	-0.54	-0.77**
CCNA_02552	*clpS*, Clp protease adaptor protein	-0.14	-1.39**	-1.99**	-0.54	1.19**
CCNA_02553	*clpA*, Clp protease ATP-binding subunit	0.21	-1.31**	-1.33**	-0.36	1.85**
CCNA_02140	*fliM*, flagellar motor switch protein	-1.14**	-1.03**	-0.58*	-0.90	-0.77**
CCNA_01067	*rsaF$_a$*, type I secretion outer membrane protein	-0.24	-1.03**	-0.91**	-0.37	1.54**
CCNA_01061	*rsaE*, type I secretion adaptor protein RsaE	-0.06	-1.32**	0.23	0.03	2.46**
CCNA_01622	ppGpp hydrolase-synthetase RelA/SpoT	-0.37	-0.98**	-0.12	-0.25	-0.02

936 * p-value < 0.05.
937 ** p-value < 0.01.
938